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Rhombomere of origin determines autonomous versus environmentally regulated expression of *Hoxa3* in the avian embryo

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SUMMARY

We have investigated the pattern and regulation of *Hoxa3* expression in the hindbrain and associated neural crest cells in the chick embryo, using whole mount in situ hybridization in conjunction with DiI labeling of neural crest cells and microsurgical manipulations. *Hoxa3* is expressed in the neural plate and later in the neural tube with a rostral border of expression corresponding to the boundary between rhombomeres (r) 4 and 5. Initial expression is diffuse and becomes sharp after boundary formation. *Hoxa3* exhibits uniform expression within r5 after formation of rhombomeric borders. Cell marking experiments reveal that neural crest cells migrating caudally, but not rostrally, from r5 and caudally from r6 express *Hoxa3* in the normal embryo. Results from transposition experiments demonstrate that expression of *Hoxa3* in r5 neural crest cells is not strictly cell-autonomous. When r5 is transposed with r4 by rostrocaudal rotation of the rhombomeres, *Hoxa3* is expressed in cells migrating lateral to transposed r5 and for a short time, in condensing ganglia, but not by neural crest within the second branchial arch. Since DiI-labeled cells from transposed r5 are present in the second arch, *Hoxa3*-expressing neural crest cells from r5 appear to down-regulate their *Hoxa3* expression in their new environment. In contrast, when r6 is transposed to the position of r4 after boundary formation, *Hoxa3* is maintained in both migrating neural crest cells and those positioned within the second branchial arch and associated ganglia. These results suggest that *Hoxa3* expression is cell-autonomous in r6 and its associated neural crest. Our results suggest that neural crest cells expressing the same *Hox* gene are not equivalent; they respond differently to environmental signals and exhibit distinct degrees of cell autonomy depending upon their rhombomere of origin.

Key words: neural crest, hindbrain, *Hox* gene, chick, rhombomere

INTRODUCTION

Both invertebrate and vertebrate embryos exhibit characteristic patterns of segmental organization during development of the body plan. For example, in *Drosophila* embryos, groups of cells organize into a series of segments and parasegments along the body axis. Individual segments express distinct Hox genes that will later determine the developmental fate of the segment (Akam, 1989). Although segmentation in the early vertebrate embryo is less obvious, the developing nervous system is overtly segmented in the region of the hindbrain, which consists of eight morphologically distinct units called rhombomeres (Vaage, 1969; Lumsden and Keynes, 1989). These rhombomeres share developmental characteristics with the body segments of *Drosophila* in that cells within a given segment tend to be restricted to a certain fate and generally do not cross rhombomere borders (Ingham and Martinez Arias, 1992; Fraser et al., 1990 but see Birgbauer and Fraser, 1994). In addition, there are notable cellular and molecular manifestations of segmentation in the hindbrain. For example, the cranial nerves develop and exit the hindbrain with a two-rhombomere perodicity, emanating only from the even-numbered rhombomeres (Keynes and Lumsden, 1990). Neural crest cells also display a segmental pattern of migration in the hindbrain, contributing to three distinct streams that populate the three branchial arches (Lumsden et al., 1991; Sechrist et al., 1993). The first stream is composed of neural crest cells from r1, 2 and 3 that migrate lateral to r2 to populate the first branchial arch. The second stream comprises cells from r3, 4 and 5 that migrate lateral to r4 and populate the second branchial arch. The third stream, composed of neural crest cells from r5, 6 and 7, migrates caudal to the otic vesicle and invades the third branchial arch.

Coupled to the morphological segmentation, there are a large array of transcription factors whose rostral boundaries of expression correspond strictly with rhombomere borders. These factors provide clues to the underlying molecular processes of segmentation (Wilkinson et al., 1989a,b; Hunt et

al., 1991a,b). A good example is the four *Hox* gene clusters, which are the vertebrate homologs of the *Drosophila* HOM-C homeotic selector genes (Akam, 1989; McGinnis and Krumlauf, 1992). Within the hindbrain, the expression of the genes displays a two-segment pattern which is colinear with the order of the genes along the chromosome. Genetic experiments in mice have demonstrated that these genes play an important role in rhombomere patterning. Loss of function mutations in *Hoxa1* lead to rhombomeric abnormalities (Mark et al., 1993; Dolle et al., 1993; Carpenter et al., 1993) and ectopic expression of *Hoxa1* induces the transformation of r2 to an r4 identity (Zhang et al., 1994). Therefore, similar to their invertebrate counterparts, the *Hox* genes appear to have a function in the specification of segmental identities. Similarly, outside of the hindbrain, *Hox* gene expression patterns are colinear in the branchial arches (neural crest, surface ectoderm and paraxial mesoderm) and align with the hindbrain. It has been suggested that a combination of *Hox* genes expressed by the migrating neural crest may pattern the regional identity of certain craniofacial structures (Hunt et al., 1991b; Noden, 1983). In support of this, one of the most common abnormalities in mouse *Hox* mutants are craniofacial defects (reviewed by Krumlauf, 1994).

Are the patterns of *Hox* gene expression in rhombomeres and neural crest independent of each other and regulated in a cell-autonomous manner or are they influenced by the local environment? Because neural crest cells arise from the rhombomeres and migrate to particular branchial arches, the combination of *Hox* genes critical for regulation of regional identity may be directly established within the rhombomeric segments and translocated by migrating cells to the periphery. Some grafting experiments suggest such autonomy. When Noden transplanted neural plates containing prospective 'first arch' neural crest cells to the level of the neural tube normally contributing to the second branchial arch (Noden, 1983), first arch structures formed at the level of the second branchial arch. This suggests that neural crest cells arising from the neural plate possess a pre-pattern and differentiate according to a positional identity that was fixed prior to transplantation. In support of this, ectopic grafting experiments have shown that the rhombomeric expression patterns of *Hoxb1* and *a2* can be cell autonomous when placed in new environments (Guthrie et al., 1992; Kuratani and Eichele, 1993; Prince and Lumsden, 1994). However, the differential expression of *Hox* genes in specific rhombomeres and their associated neural crest argues that expression is not simply transmitted in a passive manner. Rather, there is independent regulation in the neural tube and neural crest (Krumlauf, 1993; Prince and Lumsden, 1994; Nonchev et al., 1996). Genetic evidence supporting this comes from *Hoxa2* mutants, where second arch bone and connective tissue assume a first arch identity without associated changes in the rhombomeres (Rijli et al., 1993; Gendron-Maguire et al., 1993). A mechanism for this change in neural crest identity has been suggested by experiments which show that expression of *Hoxa2* in the neural crest is regulated independently of that in r4 (Nonchev et al., 1996). These experiments demonstrate that gene expression in the neural crest can be regulated independently of the neural tube but leaves open the question of whether *Hox* expression in neural crest cells is mediated cell autonomously and/or by environmental signals. Furthermore, there may differences in the neural crest arising from particular rhombomeres; for the case of *Krox 20*, there is evidence suggesting that there may be independent regulation within neural crest cells arising from r5 versus r6 (Nieto et al., 1995).

To address this question, we have examined the expression pattern of *Hoxa3* in both normal and experimentally transposed rhombomeres and their associated neural crest cells in chick embryos. In this study, we use whole mount in situ hybridization in conjunction with DiI labeling of neural crest cells to investigate the *Hoxa3* expression patterns as well as neural crest migratory pathways in the hindbrain and branchial arches. Transposition experiments show that the neural crest arising from r5 is not strictly cell-autonomous with respect to *Hoxa3* expression. In contrast, *Hoxa3* expression is maintained in r6 neural crest cells after transplantation to a new axial environment. Our results suggest that there may be differences, dependent upon the axial level of origin, in the response of neural crest cells to environmental signals.

MATERIALS AND METHODS

Embryos

White Leghorn chicken eggs were obtained from a local supplier (Hy-Line International), and incubated at 37°C until approximately stages 8 to 11 (4- to12-somite stage; ss) of development (Hamburger and Hamilton, 1951) for microsurgical manipulations and stages 6-29 for in situ hybridization. For grafting experiments, eggs were rinsed with 70% alcohol and 3 ml of albumin was removed prior to cutting a window through each shell, allowing access to the embryo. A solution of 10% India Ink (Pelikan Fount) in Howard Ringer's solution was applied below the blastoderm to visualize the embryo.

Microsurgery and DiI labeling

A slit was made in the vitelline membrane with a fine tungsten needle to expose the embryo. Fine glass micropipettes were backloaded with DiI (1,1-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate; 0.5% stock solution in 100% ethanol) and small amounts of DiI were pressure injected into the dorsal portion of the neural tube into a particular rhombomere. The characteristic undulations of the hindbrain were used as landmarks to distinguish rhombomeres from one another. Fine glass needles were then used to carefully excise either rhombomeres 4 and 5 or rhombomeres 4 through 6, including the surface ectoderm, separating these regions from the surrounding mesoderm. The excised sections were then rotated 180°, reversing their rostrocaudal polarity and maintaining dorsoventral polarity, and inserted back into the hindbrain. The majority of embryos were operated on at the 10- to 11-somite stage (stage 10) shortly after formation of rhombomere boundaries. One group of experiments was done at the 7- to 9-somite stage (stage 9), prior to the formation of borders. In some experiments, a focal injection of DiI was made into either r5 or r6. The orientation of the grafted tissue was ascertained either by eye or by using the dot of DiI as a marker for a specific rhombomere. The egg was then sealed with adhesive tape and incubated at 37°C for 16-48 hours to allow for graft healing and subsequent development.

Photoconversion of DiI

DiI-labeled embryos ranging from stages 15 to 19 of development (Hamburger and Hamilton, 1951) were harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 4°C overnight. The embryos were photographed and then prepared for photoconversion of the DiI by rinsing twice in PBS followed by 0.1 M Tris-HCl (pH 7.4) for 30 minutes at 4°C (Nieto et al., 1995). The Tris-HCl solution was then replaced with a solution of 1 mg 3,3′-diaminobenzidine tetrahydrochlorate (DAB)/ml 0.1 M Tris-HCl and

incubated at 4ºC for 30 minutes. The embryo was placed in a welled slide in excess fresh DAB solution. The DiI-labeled rhombomere with its associated neural crest was then positioned over a 40× objective of a Zeiss microscope equipped with fluorescence optics. The DiI was then photoconverted to a brown precipitate over a period of 10-15 minutes. Care was taken not to deplete the fluorescence signal, so as not to block the signal for in situ hybridization. After a brownish precipitate was detected, the embryos were transferred immediately to 0.1 M Tris-HCl for 30 minutes to halt the enzymatic reaction, and washed twice with PBS. The embryos were dehydrated through a series of methanol washes (25%, 50%, 75%, 100%) and stored at –20°C.

Whole mount in situ hybridization

For use as a probe, a 900 bp *Hoxa3* cDNA fragment was cloned into pSK (Stratagene). The antisense digoxigenin riboprobe was synthesized by linearizing the vector with *Bam*HI followed by T7 transcription. The digoxigenin-labeled probe was then used for whole-mount in situ hybridization, essentially as described by Wilkinson and Nieto (1993).

Sectioning and mounting of embryos

After in situ hybridization, embryos were prepared for cryostat sectioning by rehydrating them through successive washes of PBS/methanol (pH 7.4; 100%, 75%, 50%, 25%) and then placed into PBS. The embryos were then placed sequentially in 5% and 15% sucrose in PBS for 2-4 hours at 4°C. Embryos were embedded in 15% sucrose and 7.5% gelatin for 2-4 hours at 37°C, followed by fresh gelatin at 4°C for 30 minutes. The embryos were then frozen in liquid nitrogen, allowed to equilibrate at –25°C and cryosectioned at 30 μm. Sections were affixed to slides, coverslipped with gelmount and stored at 4°C.

To visualize the expression pattern of *Hoxa3* from a lateral view, the forebrain and trunk of the embryo were dissected away in PBS with a fine tungsten needle. The remaining embryo was pinned in the midbrain region ventral-side up for stability and bisected at the ventral midline. The embryo was then placed in a well slide with excess PBS and photographed using either an Olympus Vanox or a Zeiss SV6 microscope.

RESULTS

Cell marking analysis of *Hoxa3* expression in neural crest

In mouse embryos, the rostral border of expression of *Hoxa3* occurs at the boundary between rhombomeres (r) 4 and 5. In addition, *Hoxa3* is expressed by migrating neural crest cells and those

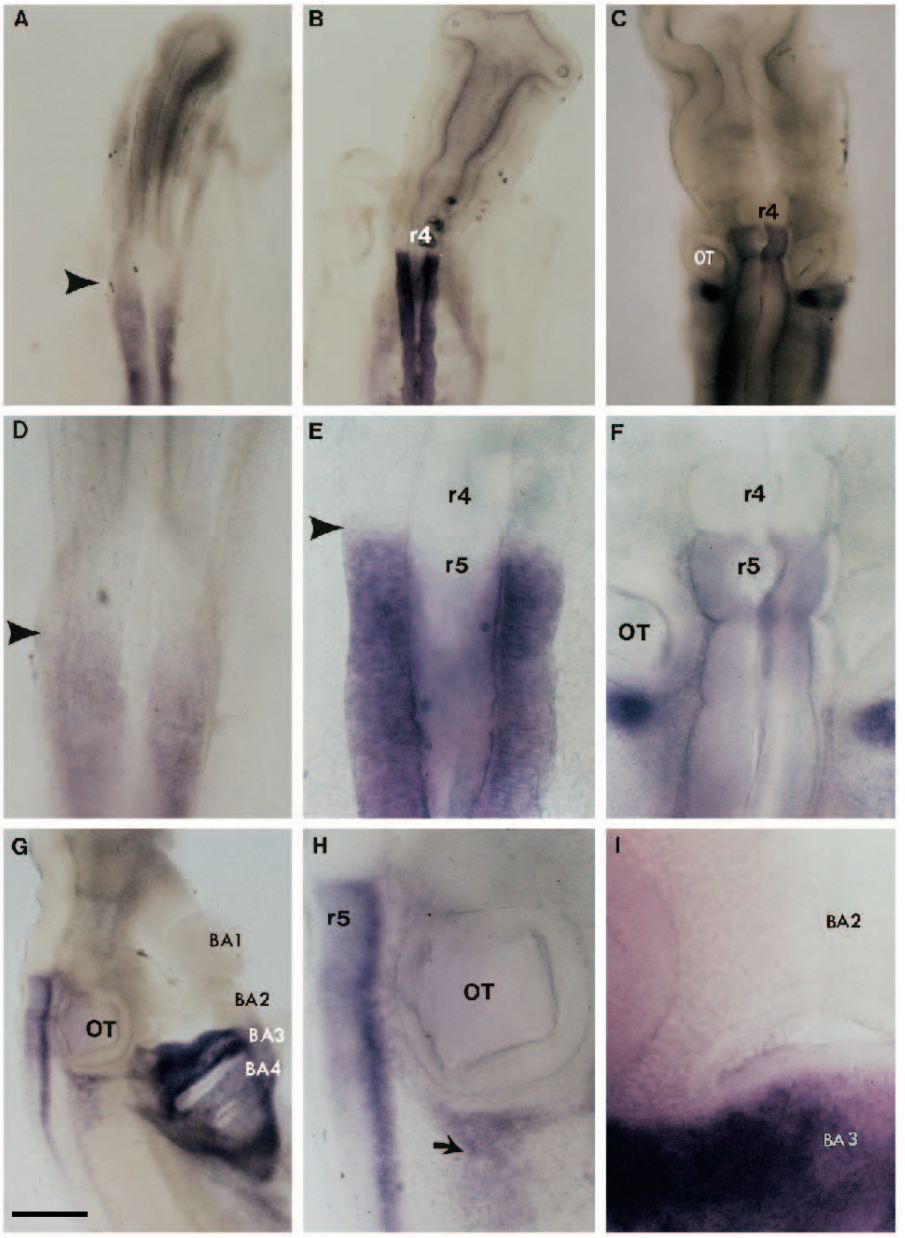

Fig. 1. *Hoxa3* in normal embryos (5ss to st. 18). (A-C) Low magnifications and corresponding higher magnifications (D-F) of *Hoxa3* expression in 5ss (A,D), 13ss (B,E) and st. 14 embryos (C,F), respectively. (A,D) Prior to boundary formation at the 5ss (stage 8), *Hoxa3* expression is diffuse in the open neural plate (folds), with the rostral limit of expression caudal to the level where the future boundary between r4 and r5 will develop (arrowhead). (B,E) As illustrated in this 13-somite embryo, the neural tube has closed in the hindbrain region by the 10-13ss (stage 10-11) and several rhombomere boundaries are clearly distinguishable, including r2/3, r3/4, and r4/5. At this time, *Hoxa3* expression is confined to the neural tube and its rostral limit of expression corresponds precisely to the r4/5 boundary (arrowhead). The intensity of staining within the neural tube appears to have increased and the rostral border appears more distinct compared to earlier stages. (C,F) By stage 14, the staining intensity of *Hoxa3* becomes much higher in r5 and r6 than in more caudal regions of the neuraxis. Neural crest cells migrating caudal to the otic vesicle (OT) express high levels of *Hoxa3* during the course of their migration and early ganglion formation. (G,H) By stage 16-18, *Hoxa3* expression is maintained in the neural tube and is present in neural crest cells migrating caudal to the otic vesicle. In this stage 18 embryo, *Hoxa3*-expressing neural crest cells have migrated to the branchial arches (BA) 3 and 4, but not 2. Some condense to form the fused proximal ganglia of the IX and X cranial nerves (arrow in H), caudal to the otic vesicle. (I) This high magnification view of the branchial arches in a stage 17 embryo shows that *Hoxa3*-positive cells are present in BA3 but not BA2. Scale bar: A,B,C,G, 105 μm; D,E,F,H, 210 μm; I, 420 μm.

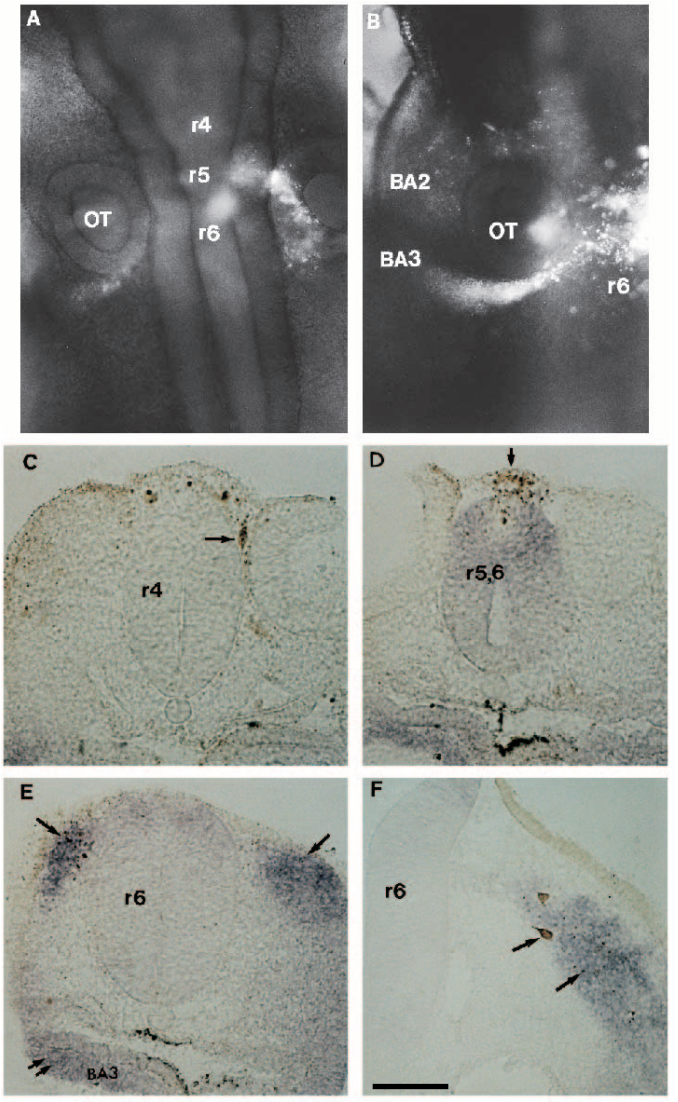

Fig. 2. DiI-labeling of rhombomere 5 in combination with in situ hybridization using a *Hoxa3* cDNA probe. (A,B) Whole-mount view of an embryo that received an injection of DiI into the caudal portion of r5 at the 10ss and was fixed at stage 17; seen with fluorescence plus phase optics (A) or fluorescence optics alone (B). A large stream of DiI-labeled cells emerging from r5 migrate caudal to the otic vesicle, (OT) en route to the third branchial arch (BA3). In addition, some labeled cells have migrated rostrally above the otic vesicle, moving toward BA2. The out of focus DiI-signal within the neural tube is fluorescent 'bloom' emanating from a discrete spot of DiI in r5. (C-E) Transverse sections through the embryo pictured in A and B after photoconversion of the DiI. At the level of caudal r4 (C) the neural tube is unlabeled, but DiI-labeled neural crest cells (arrow) can be seen above and lateral to the neural tube. Just caudal to the primary injection site (D), DiI-labeled cells (arrow) are localized within and above the neural tube. At the level of r6 (E), DiI-labeled cells are present in the prominent vagal stream of *Hoxa3*-expressing cells (arrows) that migrate caudal to the otic vesicle. (F) A similar embryo labeled at the 10ss and fixed and processed at stage 18, has DiI-labeled cells (arrows) in a *Hoxa3*-positive aggregate of cells (i.e. IX and X cranial nerve complex) caudal to the otic vesicle. Double arrows mark *Hoxa3*-reactivity in branchial arch 3; no DiI-labeled cells are present in this section. Scale bar: A-E, 75 μm; F, 150 μm.

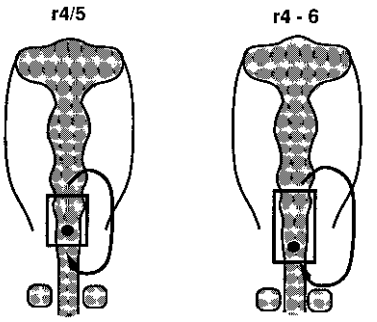

Fig. 3. Schematic diagram illustrating the operation of rhombomere rotation of r5 to the position of r4 (r4/5 transposition) and r6 to the position of r4 (r4-6 transposition). The dark spots indicate sites of DiI injection into r5 and r6, on the left and right, respectively.

localized within branchial arches 3 and 4 (Hunt et al., 1991a). To study the relationship of *Hoxa3* to migrating neural crest cells in the hindbrain, we have analyzed its expression in the developing chick embryos using a 900 bp cDNA fragment of chick *Hoxa3* as a probe.

At stage 6, *Hoxa3* expression was seen in the neural plate as a broad band in the presumptive hindbrain. By the 5 somite stage (stage 8-9), diffuse *Hoxa3* expression was seen in portions of the open neural plate with the exception of the midline (Fig. 1A,D). The rostral limit of expression was diffuse and approximately corresponded to the level where the future boundary between r4 and r5 will develop. By stage 10 (10-somite stage), the neural tube had closed in the hindbrain region and several rhombomere boundaries were clearly distinguishable. From this time onwards (stages 11-29), *Hoxa3* expression was prominent in the neural tube and its rostral limit of expression corresponded precisely to the r4/5 boundary (Fig. 1B,E). By stage 14, the staining intensity of *Hoxa3* became much higher in r5 and r6 than in more caudal regions of the neuraxis. Neural crest cells migrating caudal to the otic vesicle expressed high levels of *Hoxa3*, both during the course of their migration (Fig. 1C,F), and when they reached their destination in the fused IXth and Xth ganglia and the third and fourth branchial arches at stages 16-18 (Fig. 1G-I).

By combining cell marking techniques with in situ hybridization (Izpisua-Belmonte et al., 1993), it is possible to determine the site of origin of cells expressing a particular gene in the hindbrain (Nieto et al., 1995). To determine the rhombomere of origin of the migrating *Hoxa3*-expressing cells, we performed focal injections of DiI into the dorsal neural epithelium of r5 or r6 (Sechrist et al., 1993). Embryos were labeled at the 8 to 12 somite stage and allowed to develop to stages 14-19. After fixation, the DiI was photoconverted to an oxidized diaminobenzidine (DAB) precipitate. This was followed by in situ hybridization to visualize the *Hoxa3*-expressing cells. The photoconversion process was not carried to completion to avoid masking the hybridization signal; thus, the photoconverted signal underestimates the number and intensity of the DiI-labeled cells. Embryos were serially sectioned to examine the distribution of DiI-labeled and *Hoxa3*-expressing neural crest cells.

We observed that DiI-labeled cells from r5 (n=12; Table 1) migrated both rostral and caudal to the otic vesicle (Fig. 2A,B), in accordance with our previous work (Sechrist et al., 1993; Birgbauer et al., 1995). In serial sections through these

Table 1.

Manipulation	*n*	Stage of operation (ss)	Stage of fixation (st)	BA2 A3+	BA2 DiI+	BA3 A3+	BA3 DiI+
r4 injection only	4	10-11	16-17	0	4	4	0
r5 injection only	12	10-11	15-18	0	2	2	2
						8	8*
r6 injection only	6	10-11	16-17	0	0	6	6
r4-5 rotation	33	10-11	13-24	0		33	
r4-5 rotation, DiI in r4	4	10-11	16-17	0	4	4	4*
r4-5 rotation, DiI in r5	17	10-11	15-19	0	17	17	3*
r4-6 rotation	10	7-9	15-17	6		12*	
r4-6 rotation	5	10-11	16-18	3		5*	
r4-6 rotation, DiI in r6	10	10-11	16-18	7	10	10	6*

*Number of embryos with DiI-labeled cells in both BA2 and BA3.
BA, branchial arch.

embryos after in situ hybridization and photoconversion of DiI, there was no *Hoxa3* expression in the DiI-labeled neural crest cells that migrated from r5 rostral to the otic vesicle (Fig. 2C,D). In contrast, the DiI-labeled cells migrating caudal to the otic vesicle contributed to a stream that contained broad expression of *Hoxa3* (Fig. 2E,F). Similarly, DiI-labeled cells from r6 (*n*=6) migrated caudal to the otic vesicle, joining the *Hoxa3*-positive stream (data not shown); r6 neural crest cells did not migrate rostral to the otic vesicle, though cells from r4 did (*n*=4), confirming the results of previous studies (Sechrist et al., 1993; Birgbauer et al., 1995; Nieto et al., 1995). It is difficult to determine whether an individual neural crest cell contained both the DAB precipitate and expressed *Hoxa3*. It is likely, however, that the majority of cells migrating caudal to the otic vesicle express *Hoxa3*, based on: (1) the uniform labeling of the cells migrating caudal to the otic vesicle and (2) the fact that the number of *Hoxa3*-positive cells is similar to the total number of neural crest cells in this region. Therefore, *Hoxa3* is likely to be expressed in all neural crest cells that emigrate from r6 but only in the subset of those from r5 that migrate caudal to the otic vesicle.

Hoxa3 expression in neural crest after rostrocaudal transposition of r4 and r5 or r4 through r6

The results described above show that both rhombomeres 5 and 6 contribute neural crest cells to the *Hoxa3*-positive stream migrating caudal to the otic vesicle into the third branchial arch.

Table 2.

Manipulation	*n*	Stage of operation	Stage of fixation	Number of DiI+ cells BA2	Number of DiI+ cells BA3
r5 injection only	5	10-11ss	st. 17-18	213	290
				135	224
				57	142
				25	228
				151	218
r4-5 rot DiI in r5	5	10-11ss	st. 17-18	215	0
				171	0
				134	22
				151	0
				108	0

Although r5 contributes neural crest to both the second and third branchial arches, only those cells migrating into the third arch express *Hoxa3*. A similar restriction to the expression of *Krox-20* has been observed (Nieto et al., 1995). This could indicate that neural crest cells that migrate caudal to the otic vesicle are 'predetermined' to maintain *Hoxa3* after emigrating from the neural tube and during migration into the third arch. Alternatively, interactions with the environment caudal to the otic vesicle might either induce or maintain *Hoxa3* expression in this population; the latter possibility is consistent with our previous observation that the otic vesicle exerts an influence on migrating neural crest cells (Sechrist et al., 1993). To distinguish between these possibilities, we moved r5 and r6 to new locations by 180° rostrocaudal transpositions of rhombomeres prior to the onset of neural crest migration. We excised and rotated either rhombomeres 4 and 5 (r4/5) or rhombomeres 4 through 6 (r4-6), placing either r5 or r6 into the position normally occupied by r4. Fig. 3 illustrates the types of operations performed. In some embryos, a focal injection of DiI was made into the dorsal portion of r5 for r4/5 rotations or r6 for r4-6 rotations to follow the trajectory of neural crest cells. For most experiments, rhombomere rotation was carried out just after the formation of rhombomere boundaries. Immediately after neural tube rotation, the neural tubes and surrounding tissues appeared to be separated by a small gap which healed rapidly, usually becoming integrated by 3-5 hours after the operation. Embryos were fixed at stages 13-24, by which time the graft borders were detectable in most embryos either due to the presence of a boundary or a slight mismatch in the neural tube widths at the graft/host interface. The data is summarized in Table 1.

r4/5 transposition

When r5 was transposed to the position of r4, *Hoxa3* expression was maintained within the neural tube according to its position of origin (Fig. 4). This resulted in a 'striped' appearance in the hindbrain, with r5 expressing *Hoxa3*, whereas r4 (sandwiched between r5 and r6) was devoid of *Hoxa3* expression. In all embryos examined at stages 13 to 24 (*n*=54), numerous *Hoxa3*-positive neural crest cells emerged from the neural tube and entered the adjacent mesenchyme, as if coursing laterally as a broad stream toward the 2nd branchial arch (Fig. 4A,B). However, *Hoxa3* -positive cells were never

found deep within that arch (Fig. 4C-E). This raises the possibility that *Hoxa3* is down-regulated in this neural crest population. DiI-labeling of r5 prior to r4/5 rotation (n=17 embryos) revealed that numerous neural crest cells derived from the rotated rhombomeres migrated and survived within the second branchial arch, although no *Hoxa3*-positive cells were observed in this location (Table 1). This is best observed in transverse sections through these embryos (Fig. 5A). In contrast, all of these embryos have *Hoxa3* -positive cells in the third branchial arch 3 (Fig. 5B) and occasionally, DiI-labeled cells were observed in this location (3/17 embryos).

Hoxa3 expression in the neural crest cells condensing into ganglia lateral to rotated r5 transiently appeared in most embryos. However, there was a faint aggregate of *Hoxa3*-expressing cells observed in the dorsomedial portion of the VII/VIIIth ganglion just rostral to the otic vesicle and adjacent to the neural tube in some embryos (3/6) fixed at stages 20-24 after r4/5 rotation. Thus, *Hoxa3*-positive cells derived from r5 that are most distant from the branchial arch may maintain low levels of *Hoxa3* expression. In a few embryos, a stream of *Hoxa3*-reactive cells was observed in an ectopic location oriented rostrocaudally around the ventral portion of the otic vesicle, as if targeting the VII and VIIIth ganglia or moving toward the third branchial arch. This may indicate that some *Hoxa3*-expressing cells are attracted to their normal target. These results indicate that *Hoxa3* expression is not autonomously maintained in those r5 neural crest cells that migrate rostral to the otic vesicle and enter the second branchial arch but is instead dependent upon the environment. In both normal and transposed rhombomeres, r5-derived neural crest cells appear to down-regulate *Hoxa3* expression when they reach the second arch.

r4-6 transpositions

We next transposed r6 to the position of r4 to determine if the second arch environment had similar effects on *Hoxa3*-positive neural crest cells derived from it. After rotation of r4-6, the expression pattern of *Hoxa3* initially appeared similar to that observed in the r4/5 rotations. Within the neural tube, *Hoxa3* expression was maintained in r5 and r6, whereas rotated r4 was devoid of *Hoxa3* (Fig. 6A,B). *Hoxa3*-expressing neural crest cells migrated laterally from rotated r6 and r5 rostral to the otic vesicle and toward the second branchial arch. In contrast to the results observed with r4/5 rotations, numerous *Hoxa3*-positive cells were observed within the second branchial arch (observed in 16/27 embryos). Similar results were observed for operations performed before or after overt formation of the r5/6 border. In some cases, *Hoxa3*-positive neural crest cells appeared localized in the proximal portion of the arch, where the cranial ganglia form (Fig. 6B,C), but were less prominent in the distal portion of the arch. In other cases, *Hoxa3* -expressing cells were distributed throughout the proximodistal extent of the second branchial arch (Fig. 6D,E). Interestingly, these cells often were predominantly localized in the rostral half of the second branchial arch and there appeared to be a corresponding diminuition of cells in the rostral portion of the third branchial arch (Figs 5E,F, 6D,E).

When r6 was labeled with DiI prior to r4-6 rotations, DiI-positive cells were observed in the condensing ganglia and second branchial arch in 10/10 embryos (Fig. 5C,D) and in the third branchial arch in 6/10 embryos (Table 1), suggesting that some r6 neural crest cells deviate caudally toward their normal target. These results shows that r6-derived neural crest cells in the second arch behave in a cell autonomous manner with respect to *Hoxa3* expression.

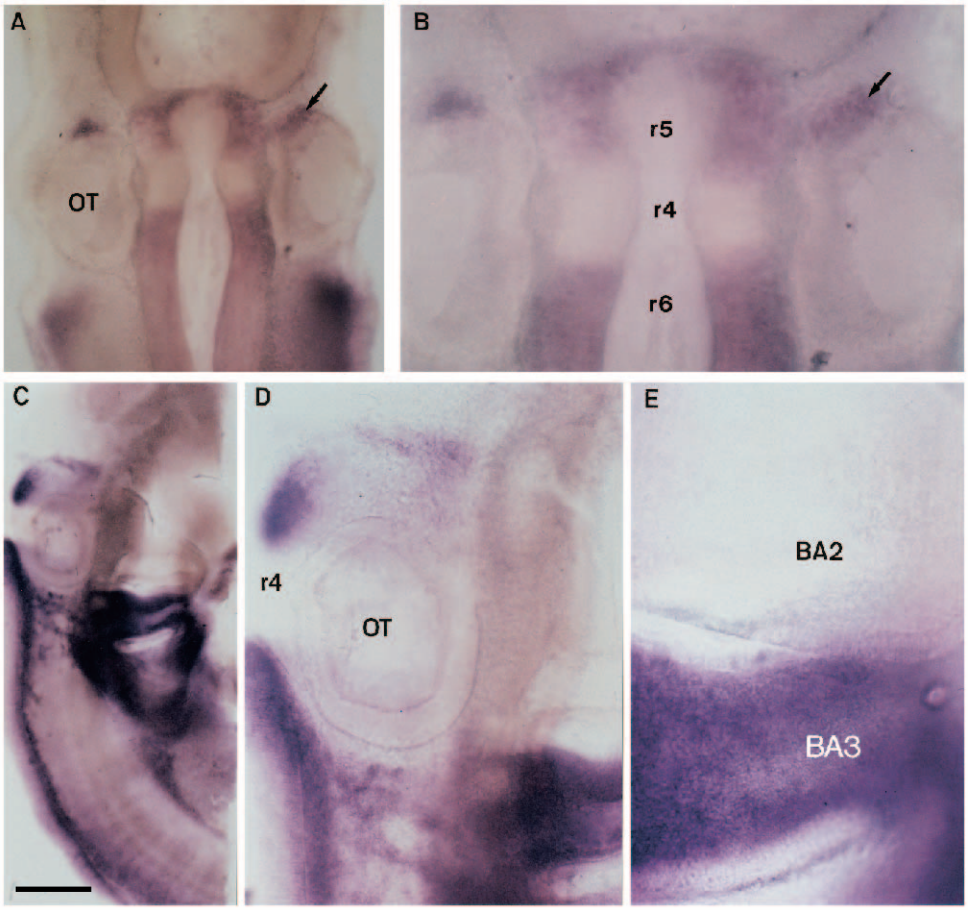

Fig. 4. Embryos after transposition of r4 /5, analyzed by in situ hybridization with a *Hoxa3* probe. (A,B) Dorsal views at low and high magnification of an embryo in which the neural tube was rotated at the 10ss and fixed at stage 17. A group of *Hoxa3*-expressing cells (arrows) emerge from rotated r5 and migrate to a position just rostral to the otic vesicle. (C,D) A side-view at low and higher magnification of another embryo rotated at the 10ss and fixed at stage 18. *Hoxa3*-expressing neural crest cells are present in the third and fourth, but not the second branchial arch, as most clearly visible in the embryo pictured in (E). OT, otic vesicle; BA2, branchial arch 2; BA3, branchial arch 3. Scale bar: A,C, 132 μm; B,D, 66 μm; E, 33 μm.

Hoxa3 expression in r5 neural crest is not cell autonomous after rostrocaudal transposition of r4 and r5

The finding that there may be some targeted migration of *Hoxa3*-expressing cells from r6 after rhombomere rotation raises the possibility that a similar phenomenon might be true for r5. Therefore, it is critical to distinguish whether the absence of *Hoxa3* expression in branchial arch 2 after r4/5 rotations comes from: (1) a down-regulation of gene expression in r5 neural crest cells or (2) a selective migration of *Hoxa3*-negative cells to branchial arch 2 and *Hoxa3*-positive cells to branchial arch 3.

To distinguish between these possibilities, we explored whether there was a mosaic distribution of *Hoxa3* neural crest precursor cells within the dorsal portion of r5 prior to neural crest cell emigration. We observed that, with the exception of the floor plate region, there appeared to be uniform expression of *Hoxa3* within the rhombomere (Fig. 7). This absence of mosaic expression in r5 eliminates the possibility that there is targeted migration of *Hoxa3*-positive precursors to branchial arch 3.

To explore further the possibility of targeted migration, we compared the numbers of DiI-labeled cells that entered branchial arches 2 and 3 in normal embryos (n=5) with those after r4/5 transposition (n=5; Table 2). For these experiments, the DiI label was not photoconverted, in order to allow accurate cell counts. In unoperated embryos, relatively similar numbers of DiI-labeled cells migrate to branchial arches 2 and 3. In contrast, the vast majority of r5 neural crest cells migrate to branchial arch 2 after r4/5 rotation. In fact, only in 1/5 embryos were some DiI-labeled cells observed in branchial arch 3. These results rule out the possibility that the apparent down-regulation of *Hoxa3* could be due to targeted migration to the third branchial arch in the vast majority of embryos.

DISCUSSION

As a step towards understanding the role that *Hox* genes may play in the development of the hindbrain-associated neural crest, we have examined the pattern of expression of *Hoxa3* in the neural crest of intact and manipulated embryos. Specifically, we wished to determine whether *Hoxa3* expression by the neural crest was influenced by environmental signals or was strictly cell-autonomous in nature.

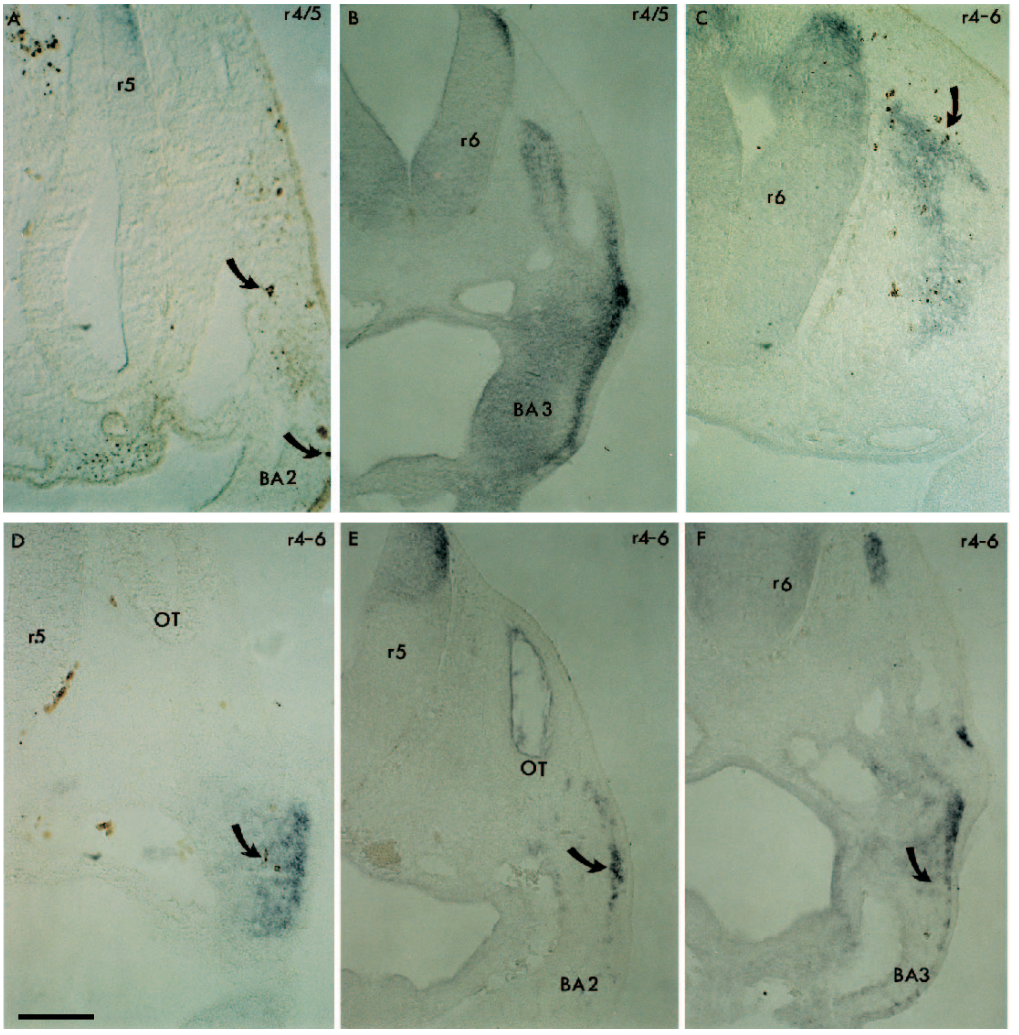

Fig. 5. Transverse sections through embryos after transposition of r4/5 and labeling of r5 with DiI (A,B) or transposition of r4-6 after labeling of r6 with DiI (C-F). (A) Section through r5 of an embryo operated at the 10ss and fixed at stage 16 after r4/5 transposition. DiI-labeled cells (arrows) can be seen migrating toward (top arrow) and into (bottom arrow) the second branchial arch (BA2), but no *Hoxa3*-expressing cells were present within this arch. (B) A section through a stage 17 embryo at the level of r6 after r4/5 transposition. *Hoxa3*-expressing cells accumulate in the third branchial arch (BA3). (C) Section through r6 of a stage 17 embryo in which r4-6 was rotated at the 10ss. DiI-labeled cells (arrow) have emerged from rotated r6 and appear to be part of a large aggregate of *Hoxa3*-expressing cells that are present at the site of the forming proximal ganglion (i.e. VII and VIII cranial nerve complex). (D) Section at the level of r5 of a stage 17 embryo in which r4-6 was rotated at the 10ss. *Hoxa3*-expressing cells localize to the region of the epibranchial placode associated with the second branchial arch. Some of these are DiI-labeled (arrow), indicating that they arose from transposed r6. (E,F) Transverse sections through the embryo pictured in Fig. 6E,F. *Hoxa3*-positive cells (arrows) are present in branchial arch 2 (E) as well as branchial arch 3 (F). Note that the number of *Hoxa3*-expressing cells in BA3 is decreased compared to that in an embryo in which r6 was not transposed (compare with B). Scale bar: 85 μm.

Hoxa3 is expressed in a subset of r5 neural crest cells

The normal spatiotemporal pattern of expression of *Hoxa3* along the chick neuraxis appears similar to that observed in the mouse (Hunt et al., 1991a). *Hoxa3* transcripts extend rostrally to the border of r4/5, throughout the mediolateral extent of the neural tube with expression sharpening as rhombomere boundaries become distinct. DiI labeling reveals that neural crest cells emigrating from both r5 and r6 contribute to a *Hoxa3*-positive stream of cells that travels caudally to the otic vesicle. These cells eventually populate the third and fourth branchial arches. Interestingly, the population of r5 neural crest cells that travels rostral to the otic vesicle into the second branchial arch was never observed to express *Hoxa3*. Thus, although the entire r5 neuroepithelium expresses *Hoxa3*, only distinct subpopulations of r5-associated neural crest cells express the gene, suggesting that the Hox 'code' is not simply transferred in a lineal fashion to the periphery by migrating neural crest cells.

In the normal embryo, down-regulation of *Hoxa3* expression in the rostral stream occurs immediately as neural crest cells leave the neuroepithelium. This conclusion is based on the fact that we never see *Hoxa3*-negative cells within the rostral portion of r5, nor do we see *Hoxa3* expression in cells emigrating from r5. This difference in the pattern of *Hoxa3* expression in rostral versus caudal r5 neural crest generally correlates with combined lineage and expression analysis of *Krox-20* (Nieto et al., 1995). This raises the intriguing possibility that *Krox 20* regulates *Hoxa3* expression in r5 neural crest, analogous to its ability to regulate *Hoxa2* and *b2* in r3 and r5 and associated neural crest cells (Sham et al., 1993; Nonchev et al., 1996). One difference is that *Hoxa3* expression is not mosaic in r5 (Fig. 7), although *Krox-20* expression is clearly mosaic in the rostral, dorsal region (Nieto et al., 1995). This does not, however, negate the possibility that *Hoxa3* may be a target of *Krox 20* since the mosaicism of the latter is transient. Thus, *Krox 20* may be regulating continuous

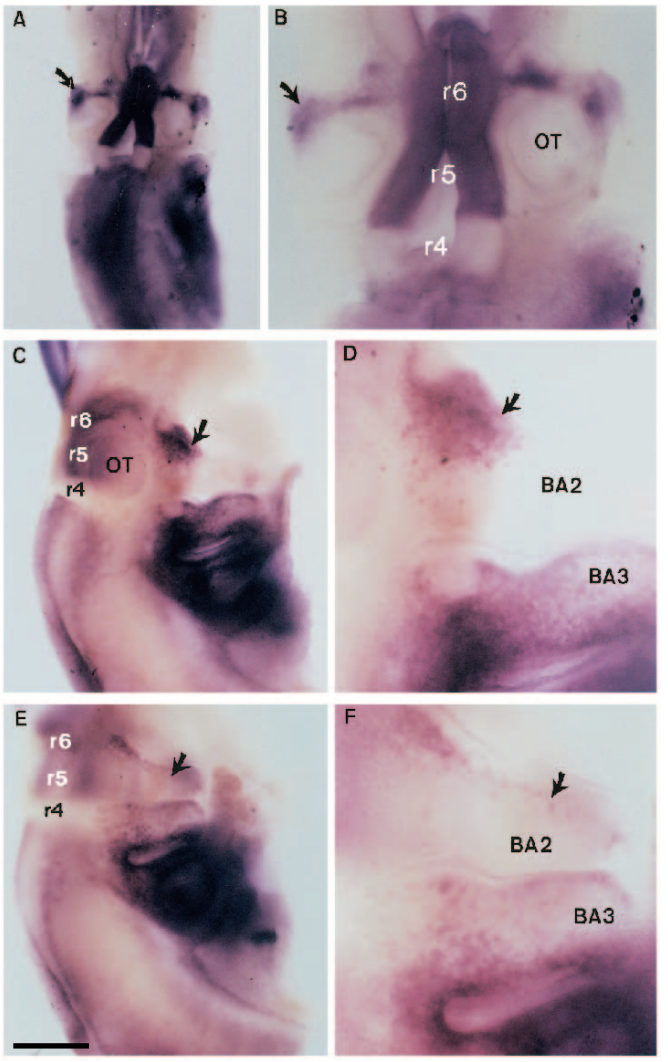

Fig. 6. Embryos after transposition of r4 through r6, analyzed by in situ hybridization with a *Hoxa3* probe. (A,B) Dorsal views at low and high magnification of an embryo in which the neural tube was rotated at the 10ss and fixed at stage 16. A prominent stream of *Hoxa3*-expressing cells (arrow) has emerged from rotated r6, migrated rostral to the otic vesicle where some cells condense to form ganglia and others migrate toward the second branchial arch. (C,D) A side-view at low and higher magnification of another embryo rotated at the 10ss and fixed at stage 18. *Hoxa3*-expressing neural crest cells (curved arrow) that emerged lateral to rotated r6 can be seen dorsal to the proximal portion of branchial arch 2 associated with the first epibranchial placode. (E,F) A side-view at low and higher magnification of another embryo rotated at the 10ss and fixed at stage 18. *Hoxa3*-expressing neural crest cells (curved arrow) that emerged lateral to rotated r6 have extended along the entire rostral portion of branchial arch 2. A concomitant decrease is seen in the normal population of *Hoxa3*-expressing cells in the rostral portion of branchial arch 3. OT, otic vesicle; BA2, branchial arch 2; BA3, branchial arch 3. Scale bar: A,C,E, 144 μm; B,D,F, 72 μm.

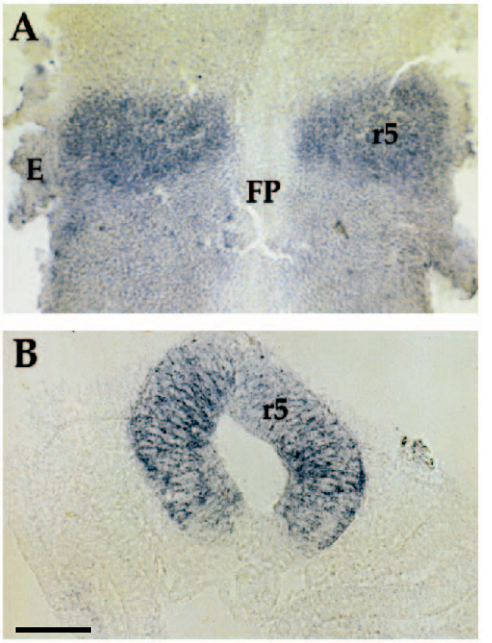

Fig. 7. Flat-mount (A) and transverse section (B) of the hindbrain in 10- to 12-somite stage embryos illustrating that *Hoxa3* shows uniform expression in rhombomere 5. (A) *Hoxa3* transcripts are distributed throughout the neuroepithelium with the exception of the floor plate (FP). The ectoderm (E) also lacks *Hoxa3* expression. (B) In transverse section through r5, the intensity of *Hoxa3* expression appears uniform throughout the rhombomere, except for the prospective floor plate region. Scale bar: A,B, 80 μm.

expression of *Hoxa3* in the hindbrain, but not its expression within the neural crest.

After transposition of r4/5, *Hoxa3* expression is only observed in migrating and aggregating neural crest cells from transposed r5 but never in cells deep within the second branchial arch. One potential explanation is that cells emigrating from the transposed r5 undergo apoptosis as proposed by Graham et al., (1993, 1994). However, the DiI-labeling of r5 demonstrates that these cells both migrate into and survive within the second branchial arch (Birgbauer et al., 1995). An alternative explanation is that the second branchial arch environment leads to down-regulation of *Hoxa3* in r5-derived neural crest cells. These events could occur directly by positive or negative influences emanating from the appropriate branchial arch. Consistent with this possibility, *Hoxa3* expression appeared to persist longest in dorsal neural crest derivatives most distant from the second branchial arch. In contrast to normal embryos, there is transient expression of *Hoxa3* in cells from transposed r5 as they migrate rostral to the otic vesicle. This may result from either priming of the cells by the adjacent environment, effects of population density, or other possibilities. It is clear, however, that the rostrally migrating cells from either normal or transposed r5 do not maintain *Hoxa3* expression in the second branchial arch or associated ganglia.

Although we favor the notion that the majority of neural crest cells from transposed r5 down-regulate *Hoxa3* in an ectopic environment, a small number of transposed cells selectively migrate to their appropriate site. Occassionally, we observed neural crest cells on the ventral portion of the otic vesicle, oriented as though they were migrating toward their normal target, the third branchial arch, after r4/5 transposition. Furthermore, we observed DiI-labeled r5 cells in the third branchial arch in 3/17 embryos after this operation. Therefore, in addition to environmentally dependent *Hox* regulation, there is likely to be some selective migration of *Hoxa3*-expressing neural crest cells from r5. One interesting possibility is that an attractive signal, perhaps displayed by the otic vesicle or third branchial arch, encourages these ectopically located *Hoxa3*-positive cells to migrate toward their normal location. In support of this idea, our previous studies have shown that the otic vesicle selectively attracts migrating neural crest cells (Sechrist et al., 1993).

r6 neural crest cells behave differently from r5 neural crest after transposition

The above results might be taken to indicate that the second branchial arch has the ability to down-regulate all neural crest expression of *Hoxa3*. However, after transposition of r6 to the position of r4, *Hoxa3*-positive cells migrate from r6 and maintain a strong level of *Hoxa3* expression after entering the second branchial arch in the majority of embryos. These results indicate that *Hoxa3* expression is largely cell-autonomous in r6-derived neural crest and not influenced by the new surroundings. This behavior is distinct from that observed for r5 neural crest. One likely possibility is that this reflects differential regulation of gene expression which is specific in neural crest cells derived from different rhombomeres. Whether these r6 neural crest cells now contribute toward second branchial arch structures or tissues patterned by branchial arch 3 is not known, as our embryos were harvested at stages before such contribution could be assayed. Rotations of presumptive r4-6 at younger stages (7-9ss), before formation of the r5/6 rhombomere border, yielded similar results to those done after borders form, suggesting that *Hoxa3* becomes fixed and autonomous prior to rhombomere boundary formation.

Our results support the notion that differences exist between the associated neural crest of odd- and even-numbered rhombomeres at early stages. *Hoxa3* expression in r5 neural crest can be influenced by the environment whereas *Hoxa3* expression by r6 neural crest persists autonomously in branchial arch two. We favor the idea that the expression of *Hoxa3* in r5 and its neural crest is regulated by different elements and upstream factors than the r6 neural crest expression. Hence, the differences in cell-autonomy may reflect different signals which are utilized to mediate expression of *Hoxa3*, rather than a difference in the response of r5 and r6 neural crest to the same signal.

With respect to the hindbrain, the results of rhombomere rotation experiments provide further support for the idea that patterns of *Hox* expression can be maintained in a cell-autonomous manner in ectopic locations, as previously shown during rhombomere transplantion (Guthrie et al., 1992; Kuratani and Eichele, 1993; Prince and Lumsden, 1994). However, our neural crest data, together with recent alterations in head structures caused by gain and loss of function experiments in the mouse and the characterization of regulatory regions of *Hox* genes, indicate that a strict *Hox* code for the hindbrain and corresponding neural crest is not simply set in the early hindbrain and passively translocated to the neural crest. It is not surprising that there may be rapid regulation of *Hox* genes in both the neural tube and neural crest, since cell-marking experiments have shown that cells can rapidly cross rhombomere borders (Birgbauer and Fraser, 1994; Birgbauer et al., 1995), thus entering domains of different gene expression. Thus, the situation must be more complex and dynamic, in that the restricted domains for expression in rhombomeres and neural crest are built up by the combinatorial action of a diverse set of regulatory components, which also include roles for environmental influences. As our experiments demonstrate differences in *Hoxa3*-positive neural crest from r5 and r6, gene expression in these tissues is not always irreversibly locked in a cell-autonomous manner. These experiments focus attention on the potential roles of environmental factors such as guidance signals or specific maintenance/proliferation signals in generating differential regulation of *Hox* genes in the hindbrain and neural crest.

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